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13. ABSTRACT (Maximum 200 Words)

Ipl1p is the budding yeast member of the highly conserved Ipl1/Aurora kinase family. Ipl1p is required for chromosome biorientation and the spindle checkpoint. Previous work done in the Biggins laboratory uncovered new functions for Ipl1p in spindle disassembly and spindle orientation. Ipl1p localizes to the spindle midzone during anaphase and tracks the plus ends of the depolymerizing spindle MTs. Cells lacking Kip3p, a MT destabilizing kinesin, are delayed in spindle breakdown similar to *ipl1* mutants. It is possible that Ipl1p regulates spindle breakdown by directly regulating Kip3 activity. We found that Kip3p is phosphorylated by Ipl1p *in vitro* and is a phospho-protein *in vivo*. Mutating one of the Ipl1p consensus sites in Kip3p generates a spindle disassembly delay similar to *ipl1* and *kip3Δ* mutants. We are currently testing whether the Kip3p is phosphorylated by Ipl1p *in vivo*. In addition we have uncovered a new role for Ipl1p in spindle assembly that is independent of its other functions. Ipl1p acts in parallel with Cin8p, a kinesin-like motor protein, in the assembly of a bipolar spindle.

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Table of Contents

Introduction

The accurate propagation of genetic information from one cell generation to the next depends on the faithful segregation of chromosomes during mitosis. Rearrangement of the microtubule (MT) cytoskeleton during mitosis plays a critical role in chromosome segregation as well as other events associated with cell division. After chromosomes replicate during S phase linkage between sister chromatids is established (for review, see [1]). When the mitotic spindle forms, microtubules emanating from the spindle poles attach to chromosomes and this attachment is mediated by the kinetochore, a multiprotein complex that assembles onto centromeric DNA of each sister chromatid [2]. Incorrect MT-kinetochore attachments can lead to missegregation giving rise to aneuploidy, a hallmark of most cancers.

The focus of the Biggins laboratory is to understand the mechanisms involved in chromosome segregation using the budding yeast, *Saccharomyces cerevisiae*, as a model system. Many key components of the cell cycle machinery were originally discovered in budding yeast and are highly conserved in higher eukaryotes.

Ipl1p is the budding yeast member of the highly conserved Ipl1/Aurora protein kinase family that is implicated in oncogenesis [3,4]. Analysis of temperature sensitive alleles has shown that Ipl1p regulates at least four mitotic processes. 1. Ipl1p is required for chromosome bi-orientation as in the absence of Ipl1p function sister chromatids often attach to MTs from the same spindle pole body (SPB) [5,6]. 2. Ipl1p is required for the activation of the spindle checkpoint when kinetochores are not under tension [7]. The spindle checkpoint is a surveillance mechanism that halts the cell cycle in metaphase in response to improperly attached or unattached kinetochores. 3. Ipl1p is required for the prompt disassembly of the spindle during telophase [8]. 4. Finally, Ipl1p plays a role in orienting the spindle along the mother-bud axis [8]. Thus Ipl1p appears to be a master regulator that coordinates many cell cycle events and it may do so by a common mechanism involving regulation of MT dynamics.

Body

Identification of the Ipl1p activating kinase

Using a variety of approaches our lab has been unable to identify the activating kinase for Ipl1p. Ipl1p displays dynamic localization during the cell cycle [8]. We are currently testing the hypothesis that the various functions of Ipl1p may be regulated by its sub-cellular location during the cell cycle instead of an upstream activating kinase.

I have started analyzing the localization of Ipl1p tagged with a triple green fluorescent protein tag in the absence of various kinetochore, kinesin-like motor, and microtubule associated proteins. So far I have observed altered localization of Ipl1p only in *ndc10* mutant cells. Since the *ndc10* mutation abolishes the entire kinetochore, I have yet to determine if the altered localization is a non-specific effect of the mutation. I am currently investigating the localization of Ipl1p in other alleles of *ndc10*, which do not abolish the entire kinetochore in order to address this question.

Identification of Ipl1p substrates

Previous studies in the Biggins lab uncovered a novel function for Ipl1p in spindle microtubule disassembly. Moreover, these studies showed this function is independent of its function in chromosome segregation [8]. Spindle MT disassembly is delayed in *ipl1* mutant cells by ~6 min leading to a 42% increase in the duration of anaphase B [8]. Ipl1p localizes to the spindle midzone at the time of spindle breakdown. The spindle midzone is the region of overlap between antiparallel MTs emanating from the spindle poles. Spindle breakdown occurs due to the destabilization of the inter-digitating MT plus ends at the midzone. A discrete number of proteins localize to the spindle midzone at the same time as Ipl1p. Some of these proteins are known to affect MT dynamics. My hypothesis is that Ipl1p promotes spindle disassembly by regulating one or more of the proteins that localize to the spindle midzone. My goal has been to identify this downstream target(s) of Ipl1p that is involved in spindle disassembly. An excellent candidate is Kip3p.

Kip3p belongs to the KinI family of kinesin-like motors and members of the KinI family have been shown to have microtubule destabilizing activity [9]. Buvelot *et al* showed that *kip3Δ* cells have the same spindle disassembly delay as *ipl1* mutants [8]. Ipl1p and Kip3p probably act in the same pathway as the double mutant cells exhibit the same delay as either single mutant. It is therefore possible that Ipl1p positively regulates Kip3p activity via phosphorylation. I have determined that Kip3p is a substrate for Ipl1p *in vitro* (Figure 1). I have also shown that Kip3p is a phospho-protein *in vivo* (Figure 1).

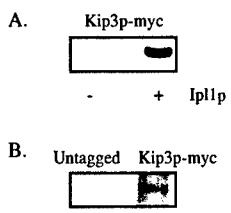

Figure 1. Kip3p is an Ipl1p substrate *in vitro* and is a phosphoprotein *in vivo*.
A. Epitope tagged Kip3p was immunoprecipitated from yeast and incubated with radioactive ^{32}P-ATP with (+) or without (-) recombinant Ipl1p.
B. Epitope tagged or untagged Kip3p was immunoprecipitated from yeast cells that were subject to orthophosphate labeling. Autoradiograph is shown.

Kip3p contains two Ipl1p consensus sites, which I mutated from serine to alanine. Only one of these point mutants (Kip3pS74A) has a spindle disassembly delay similar to the *ipl1* and *kip3Δ* mutant cells (Figure 2). I am currently testing if serine 74 is phosphorylated *in vivo* and whether this phosphorylation is dependent on Ipl1p. If Kip3p is an Ipl1p target I plan to test if the Kip3pS74A mutant has reduced MT depolymerizing activity *in vitro*. These experiments may provide us with a general mechanism for how Ipl1p regulates microtubule dynamics in the cell.

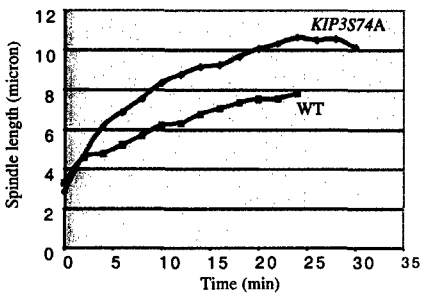

Figure 2. Live microscopy was performed on wild-type and *kip3S74A* mutant cells containing Tub1-GFP that were released from G1 at 23 C. Eight z sections at 0.4 μm intervals were acquired every 40 seconds. The spindle length at each time point was measured from the time of spindle elongation. The averages of 10 cells for each strain are graphed. Spindles disassemble in wild-type cells 24 min after anaphase B initiation, whereas *kip3S74A* mutant cells breakdown spindles after 32 min.

In addition, I have also analyzed spindle disassembly in mutant cells that lack other midzone proteins. These include Ndc10p and Ase1p, which also localize to the spindle midzone in anaphase. Neither *ndc10* nor *ase1* mutant cells have a spindle disassembly delay similar to *ipl1* mutants suggesting that Ndc10p and Ase1p are not Ipl1p targets in spindle breakdown. I am currently testing other candidate mutants for defects in spindle disassembly.

Analysis of the role of Ipl1p in spindle function

IPL1 displays genetic interactions with various genes required for spindle integrity including *CIN8* and *BIM1*. The allele *ipl1-315* was discovered in a screen for genes required for viability in the absence of the kinesin-like motor protein, Cin8p [10]. *Ipl1-315* mutant cells are not temperature sensitive, suggesting that they are not defective in chromosome segregation, the only essential function of Ipl1p (Figure 3). I have confirmed this by a sectoring assay that monitors chromosome loss. The mutation does render the kinase defective in kinase activity (Figure 3). However, Ipl1p-315 is proficient in the spindle checkpoint, chromosome bi-orientation, spindle orientation and disassembly (data not shown).

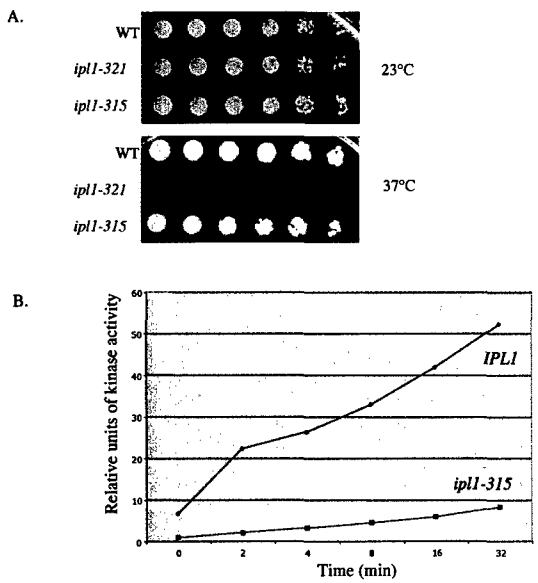

Figure 3. Analysis of *ipl1-315* mutant phenotype and kinase activity. A. *ipl1-315* mutant cells are functional at 37°C. Five-fold serial dilutions of wild-type, *ipl1-321*, *ipl1-315* cells were plated at 23° and 37°C. B. Ipl1p-315 has reduced kinase activity. Epitope tagged wild-type Ipl1p or Ipl1p-315 were immunoprecipitated from yeast cells. In vitro kinase assays were performed with one half of immunoprecipiated Ipl1p and recombinant Cse4p as substrate in the presence of ^{32}P-ATP. The other half of immunoprecipitated protein was subjected to quantitative western blotting. Graph shows kinase activity of wild-type Ipl1p or Ipl1p-315 over time after normalization to the amount of protein immunoprecipitated.

In order to determine the previously unidentified Ipl1p function, I analyzed the terminal phenotype of the *cin8 ipl1* double mutants. To do this, I have utilized a conditional degron allele

of *CIN8* that I will refer to as *cin8-d*. Cin8p-d is ubiquitinated and targeted for degradation by the ubiquitin protein-ligase Ubr1p. *UBR1* is under the control of the inducible galactose promoter allowing the degradation of Cin8p-d by switching the carbon source to galactose and growing the cells at 30 °C. *Cin8-d ipl1* mutant cells are inviable at this intermediate temperature. Since Cin8p is normally required for bipolar spindle assembly, it is possible that Ipl1p participates in spindle assembly [11]. I therefore analyzed spindle morphology in wild-type, *cin8-d* and *cin8-d ipl1-315* cells. Cells containing fluorescently labeled tubulin were synchronized in G1 and released to 30 °C. The majority of wild-type cells went through spindle elongation and disassembled their spindles 120' after release (Figure 4). However, at that time, only 15% of the *cin8-d ipl1* double mutant cells had gone through spindle elongation (Figure 4). The majority of the *cin8-d ipl1-315* cells appeared to have mono-polar spindles. This suggests that Ipl1p and Cin8p may have overlapping roles in bipolar spindle assembly.

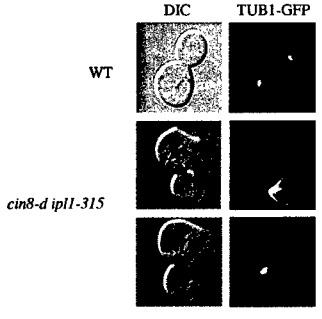

Figure 4. Ipl1p-315 acts in parallel with Cin8p in the assembly of a bipolar spindle. Wild-type and *cin8-d ipl1-315* cells containing tubulin-GFP were arrested in G1 with the mating pheromone, α-factor, and released into galactose media in order to induce degradation of Cin8p. Samples were collected 120' after release into the cell cycle and spindles were visualized by fluorescence microscopy.

It is possible that Ipl1p regulates spindle assembly by activating Kip1p, a kinesin-like motor protein that acts in parallel to Cin8p in the assembly of a bipolar spindle. In that case the *cin8-d kip1Δ* double mutant phenotype should be similar to the *cin8-d ipl1* phenotype. I compared spindle pole body (SPB) separation in *cin8-d kip1Δ* cells to that in *cin8-d ipl1-315* cells by time-lapse microscopy. In order to visualize SPBs, a GFP tagged SPB protein (Spc42p-GFP) was used. Cells containing *SPC42-GFP* were synchronized in G1 and then released to 30 °C. 100% of the wild-type cells separated their SPBs and maintained bipolar spindles throughout the time course. On the other hand, *cin8-d* single mutants had bipolar spindles in only 63% of the cells while only 53% of the *cin8-d kip1Δ* cells assembled bipolar spindles. The bipolar spindles that did assemble collapsed several times before reassembling in both the *cin8-d* single mutants and *cin8-d kip1Δ* double mutants. Strikingly, 91% of *cin8-d ipl1-315* cells never separated their SPBs and assembled bipolar spindles. Since the *cin8-d kip1Δ* mutant phenotype is significantly different from the *cin8-d ipl1-315* phenotype, that Ipl1p functions in a different pathway from Kip1p. I am currently trying to identify the downstream target of Ipl1p involved in spindle assembly.

Key Research Accomplishments

- Kip3p, a kinesin-like motor protein, localizes to the spindle midzone at the same time as Ipl1p
- Kip3p is phosphorylated by Ipl1p *in vitro* and is a phospho-protein *in vivo*

- Mutating one of the Ipl1p consensus sites in Kip3 generates a spindle disassembly delay
- *Ndc10* and *ase1* mutants do not have a spindle breakdown defect
- Characterization of *ipl1-315* mutant cells suggests that Ipl1p has a role in spindle assembly
- Ipl1p acts in parallel with Cin8p and Kip1p in the assembly of a bipolar spindle

Reportable Outcomes

- None

Conclusions

Ipl1p localizes to the spindle midzone in anaphase and follows the plus ends of the depolymerizing spindle. Previous studies from our lab have shown that *ipl1* mutants are significantly delayed in spindle breakdown. In addition, I have also uncovered a novel role for Ipl1p in the assembly of a bipolar spindle. It is interesting to note that all the processes that Ipl1p participates in require proper regulation of MT dynamics. Motor proteins and microtubule associated proteins (MAPs) have been known to modulate MT dynamics during various cellular processes. It is possible that Ipl1p may execute all its functions by regulating activities of motor proteins and MAPs. This would provide an elegant explanation for how a single protein is able to perform a diverse set of functions.

References

1. Nasmyth, K. *Segregating sister genomes: the molecular biology of chromosome separation.* Science, 2002. **297**: 559-565
2. Hyman, A.A., and Sorger, P.K. *Structure and function of kinetochores in budding yeast.* Ann Rev Cell Dev Biol., 1995. **11**: 471-495
3. Bischoff, J. R., *et al. A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers.* EMBO J., 1998. **17**: 3052-3065
4. Bischoff, J. R., and Plowman, G.D. *The Aurora/Ipl1 kinase family: regulators of chromosome segregation and cytokinesis.* Trends Cell Biol., 1999. **9**: 454-459
5. Biggins, S., et al., *The conserved protein kinase Ipl1 regulates microtubule binding to kinetochores in budding yeast.* Genes Dev, 1999. **13**(5): p. 532-44.
6. Tanaka, T.U., et al., *Evidence that the Ipl1-Sli15 (Aurora kinase-INCENP) complex promotes chromosome bi-orientation by altering kinetochore-spindle pole connections.* Cell, 2002. **108**(3): p. 317-29.
7. Biggins, S. and A.W. Murray, *The budding yeast protein kinase Ipl1/Aurora allows the absence of tension to activate the spindle checkpoint.* Genes Dev, 2001. **15**(23): p. 3118-29.
8. Buvelot, S., et al., *The Budding Yeast Ipl1p/Aurora Protein Kinase Regulates Mitotic Spindle Disassembly.* J. Cell Biol., 2003. **160**: 329-339.
9. Desai, A., et al. *Kin I kinesins are microtubule-destabilizing enzymes.* Cell, 1999. **96**: 69-78.
10. Geiser, J.R., et al. Sacchromyces cerevisiae *genes required in the absence of the* CIN8-*encoded spindle motor act in functionally diverse mitotic pathways.* Mol. Biol. Cell., 1997. **8**: 1035-1050
11. Hoyt, M.A., et al. *Two* Sacchromyces cerevisiae *kinesin-related gene-products required for mitotic spindle assembly.* J. Cell Biol., 1992. **118**: 109-120

Appendix

Training received:

- Mentored a high school science teacher for two weeks in our laboratory in the summer of 2003.
- Attend weekly seminars held at the FHCRC and the University of Washington.
- Attend monthly Seattle Area Yeast Group Meeting.
- Passed general exam in January, 2004